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(54) Title: PROCESS FOR THE FERMENTATIVE PRODUCTION OF DEACYLATED CEPHALOSPORINS

(57) Abstract

The present invention discloses a process for the production of N-deacylated cephalosporin compounds via the fermentative production of their 7-acylated counterparts.

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Process for the fermentative production of deacylated cephalosporins

Field of the invention

The present invention relates to the field of fermentative production of N-deacylated cephalosporin compounds, such as 7-ADCA.

Background of the invention

β-Lactam antibiotics constitute the most important group of antibiotic compounds, with a long history of clinical use. Among this group, the prominent ones are the penicillins and cephalosporins. These compounds are naturally produced by the filamentous fungi *Penicillium chrysogenum* and *Acremonium chrysogenum*, respectively.

As a result of classical strain improvement techniques, the production levels of the antibiotics in *Penicillium chrysogenum* and *Acremonium chrysogenum* have increased dramatically over the past decades. With the increasing knowledge of the biosynthetic pathways leading to penicillins and cephalosporins, and the advent of recombinant DNA technology, new tools for the improvement of production strains and for the *in vivo* derivatization of the compounds have become available.

Most enzymes involved in β-lactam biosynthesis have been identified and their corresponding genes been cloned, as is decribed by Ingolia and Queener, Med. Res. Rev. 9 (1989), 245-264 (biosynthesis route and enzymes), and Aharonowitz, Cohen, and Martin, Ann. Rev. Microbiol. 46 (1992), 461-495 (gene cloning).

The first two steps in the biosynthesis of penicillin in *P. chrysogenum* are the condensation of the three amino acids L-5-amino-5-carboxypentanoic acid (L-α-aminoadipic acid) (A), L-cysteine (C) and L-valine (V) into the tripeptide LLD-ACV, followed by cyclization of this tripeptide to form isopenicillin N. This compound contains the typical β-lactam structure.

These first two steps in the biosynthesis of penicillins are common in penicillin, cephamycin and cephalosporin producing fungi and bacteria.

The third step involves the exchange of the hydrophilic D-α-aminoadipic acid side chain of isopenicillin N by L-5-amino-5-carboxypentanoic acid by the action of the enzyme acyltransferase (AT). The enzymatic exchange reaction mediated by AT takes place inside a cellular organelle, the microbody, as has been described in EP-A-0448180.

In cephalosporin-producing organisms, the third step is the isomerization of isopenicillin N to penicillin N by an epimerase, whereupon the five-membered ring structure characteristic of penicillins is expanded by the enzyme expandase to the six-membered ring characteristic of cephalosporins.

The only directly fermented penicillins of industrial importance are penicillin V and penicillin G, produced by adding the hydrophobic side chain precursors phenoxyacetic acid or phenylacetic acid, respectively, during fermentation of *P. chrysogenum*, thereby replacing the side chains of the natural β-lactams with phenoxyacetic acid or phenylacetic acid.

Cephalosporins are much more expensive than penicillins. One reason is that some cephalosporins (e.g. cephalexin) are made from penicillins by a number of chemical conversions. Cephalosporin C, by far the most important starting material in this respect, is very soluble in water at any pH, thus implying lengthy and costly isolation processes using cumbersome and expensive column technology. Cephalosporin C obtained in this way has to be converted into therapeutically used cephalosporins by a number of chemical and enzymatic conversions.

The cephalosporin intermediate 7-ADCA is currently produced by chemical derivatization of penicillin G. The necessary chemical steps to produce 7-ADCA involve the expansion of the 5-membered penicillin ring structure to a 6-membered cephalosporin ring structure.

Recently, fermentative processes have been disclosed to obtain 7-ADCA.

In EP-A-0532341 the application of an adipate (5-carboxypentanoate) feedstock was shown to result in formation of a penicillin derivative with an adipyl side chain, viz. adipyl-6-aminopenicillanic acid. This incorporation is due to the fact that the acyltransferase has a proven wide substrate specificity (Behrens *et al.*, J. Biol. Chem. 175 (1948), 751-809; Cole, Process. Biochem. 1 (1966), 334-338; Ballio *et al.*, Nature 185 (1960), 97-99). In addition, when adipate is fed to a recombinant *P. chrysogenum* strain expressing an expandase, the adipyl-6-APA is expanded to its corresponding cephalosporin derivative. Finally, the removal of the adipyl side chain is suggested, yielding 7-ADCA as a final product.

The patent application EP-A-0540210 describes a similar process for the preparation of 7-ACA, including the extra steps of converting the 3-methyl group of the ADCA ring into the 3-acetoxymethyl group of ACA.

WO95/04148 and WO95/04149 disclose a feedstock of certain thiogroup-containing dicarboxylic acids with a chain length of 6 or 7 atoms to an expandase-expressing *P. chrysogenum* strain, resulting in the incorporation of these precursors into the penicillin backbone and subsequent expansion to the corresponding 7-ADCA derivatives.

In general, it is however thought that an expandase that may provide the crucial link between penicillin N and cephalosporin biosynthesis has a narrow specificity (Maea et al.,Enzyme and Microbial Technology (1995) 17: 231-234; Baldwin et al., J. Chem. Soc. Chem. Commun. 374-375, 1987), preventing the possibility for catalysing the oxidative ring expansion of penicillin N with unnatural side chains.

It now surprisingly is found that a feedstock of dicarboxylic acids with a chain length which is longer than 7 carbon atoms produce β-lactam derivatives incorporating a side chain with a chain length of either 6 or 7 atoms.

Summary of the invention

The present invention discloses a process for the production of an N-deacylated cephalosporin compound comprising the steps of:

* fermenting a microbial strain capable of β-lactam production and expressing acyltransferase as well as expandase activity, and optionally acetyltransferase and/or hydroxylase activity, in the presence of a side chain precursor according to formula (1)

$$HOOC\text{-}X\text{-}(CH_2)_n\text{-}COOH \qquad (1)$$

wherein

n is an even number of at least 2, and

X is $(CH_2)_p\text{-}A\text{-}(CH_2)_q$, wherein

p and q each individually are 0, 1, 2, 3 or 4, and

A is CH=CH, C≡C, CHB, C=O, O, S, NH, the nitrogen optionally being substituted or the sulfur optionally being oxidized, and B is hydrogen, halogen, C_{1-3} alkoxy, hydroxyl, or optionally substituted methyl, with the proviso that p+q should be 2 or 3, when A is CH=CH or C≡C, or p+q should be 3 or 4, when A is CHB, C=O, O, S or NH,

or a salt, ester or amide thereof, said side chain precursor yielding a acyl-6-APA derivative, the acyl group having a structure according to formula (2)

$$HOOC\text{-}X\text{-}CO\text{-} \qquad (2)$$

wherein X is defined as above,

said acyl-6-APA derivative being *in situ* expanded to the corresponding acyl-7-ADCA derivative, and optionally further reacted to the acyl-7-ADAC or acyl-7-ACA derivative,

* recovering the acyl-7-cephalosporin derivative from the fermentation broth
* deacylating said acyl-7-cephalosporin derivative, and
* recovering the crystalline 7-cephalosporin compound.

Detailed description of the invention

The present invention discloses a process for the production of N-deacylated cephalosporin compounds (7-ADCA, 7-ADAC or 7-ACA) via the fermentative production of their acylated counterparts, applying a feed of novel side chain precursors.

The present invention surprisingly shows that fermentation of a microbial strain capable of β-lactam production and expressing acyltransferase as well as expandase activity in the presence of a dicarboxylic acid having a chain length which is longer than 7 atoms results in the formation of an acyl-7-ADCA derivative incorporating an acyl group with a chain length of 6 or 7 atoms, respectively.

According to the invention, additional 7-acylated cephalosporin derivatives than acyl-7-ADCA, i.e. acyl-7-ADAC or acyl-7-ACA, respectively, are produced by a microbial strain capable of β-lactam production and expressing acyltransferase as well expandase, if said microbial strain additionally expresses hydroxylase or hydroxylase plus acetyltransferase activity, respectively.

The dicarboxylic acid to be used in the process of the invention has a structure according to formula (1):

$$HOOC\text{-}X\text{-}(CH_2)_n\text{-}COOH \qquad (1)$$

wherein

n is an even number of at least 2, and

X is $(CH_2)_p$-A-$(CH_2)_q$, wherein

p and q each individually are 0, 1, 2, 3 or 4, with the proviso that p+q = 2, 3 or 4, and

A is CH=CH, C≡C, CHB, C=O, O, S, NH, the nitrogen optionally being substituted or the sulfur optionally being oxidized, and B is hydrogen, halogen, C_{1-3} alkoxy, hydroxyl, or optionally substituted methyl.

According to the invention, the fermentation of said microbial strain in the presence of a side chain precursor according to formula (1), or a salt, an ester or an amide,

thereof, results in the formation of an acyl-7-cephalosporin derivative, wherein the acyl group has a structure according to formula (2):

HOOC-X-CO- (2)

wherein X is defined as above.

To obtain an acyl-7-cephalosporin derivative with an acyl group having a chain length of 6 or 7 atoms, respectively, p+q should be 2 or 3, respectively, when A is CH=CH or C≡C, or p+q should be 3 or 4, respectively, when A is CHB, C=O, O, S, NH, the nitrogen optionally being substituted or the sulfur optionally being oxidized, and B is defined as above.

Thus, a fermentation of a microbial strain capable of β-lactam production and expressing acyltransferase as well as expandase activity in the presence of a precursor compound according to formula (1) yields an acyl-6-APA derivative with an acyl group according to formula (2), which subsequently is expanded *in situ* to yield the corresponding acyl-7-ADCA derivative. In other words, said precursor compound according to formula (1) is metabolized by the microbial strain, producing an acyl group of formula (2). Said acyl group subsequently is incorporated in the β-lactam backbone via the acyltransferase-mediated reaction.

The upper limit for the chain length of the precursor compound according to formula 1, i.e. the upper value of n, is not critical. The upper limit mainly will be determined by the efficiency by which said precursor is metabolized by the microbial strain. Conveniently, the precursor may have a longest chain length which is similar to the longest chain length of a fatty acid which still can be metabolized by the microbial strain.

In one embodiment of the invention, dicarboxylic acids are used which yield an adipyl-7-ADCA derivative upon fermentation in the presence of said dicarboxylic acid. Dicarboxylic acids suitable to yield adipyl-7-ADCA have a structure according to formula (1), wherein n is an even number of at least 2, and X

is $(CH_2)_p$-A-$(CH_2)_q$, wherein p is 1 and q is 2 and A is CH_2. Preferably, said dicarboxylic acid yielding adipyl-7-ADCA is suberic acid or sebacaic acid (n = 2 or 4, respectively).

In another embodiment of the invention, dicarboxylic acids are used which yield an acyl-7-ADCA derivative containing a thiogroup in the acylgroup according to formula (2). Dicarboxylic acids suitable to yield such acyl-7-ADCA compounds have a structure according to formula (1), wherein n is an even number of at least 2, and X is $(CH_2)_p$-A-$(CH_2)_q$, wherein A is S. Preferably, p and q are 1, 2 or 3 and p+q = 3 or 4. Most preferably, p is 1 and q is 2, or p is 2 and q is 1 or 2.

In two other embodiments of the invention, dicarboxylic acids are used which yield novel acyl-7-cephalosporin derivatives.

Firstly, dicarboxylic acids are used which yield a pimelyl-7-ADCA derivative upon fermentation in the presence of said dicarboxylic acid. Dicarboxylic acids suitable to yield pimelyl-7-ADCA have a structure according to formula (1), wherein n is an even number of at least 2, and X is $(CH_2)_p$-A-$(CH_2)_q$, wherein p and q are 2 and A is CH_2. Preferably, said dicarboxylic acid yielding pimelyl-7-ADCA is azelaic acid (n = 2).

In addition, dicarboxylic acids are used which yield an acyl-7-ADCA derivative containing an unsaturated bond in the acylgroup according to formula (2). Dicarboxylic acids suitable to yield such acyl-7-ADCA compounds have a structure according to formula (1), wherein n is an even number of at least 2, and X is $(CH_2)_p$-A-$(CH_2)_q$, wherein A is CH=CH or C≡C. Preferably, A is CH=CH and p and q both are 1. The trans isomer of the latter compound thereby is most preferred.

Microbial strains which are usable in the process of the invention are strains which are capable of β-lactam production and which express acyltransferase as well as expandase activity. Optionally, said microbial strains additionally may express hydroxylase or hydroxylase plus acetyltransferase activity. The former strains enable production of acyl-7-ADCA derivatives, whereas the latter strains enable production of acyl-7-ADAC or acyl-7-ACA derivatives.

Examples of such microbial strains include penicillin-producing strains provided with an expression cassette providing for expandase expression and cephalosporin-producing strains provided with an expression cassette providing for acyltransferase expression.

Expandase genes which conveniently are used may originate from *Acremonium chrysogenum*, *Streptomyces clavuligerus*, *Streptomyces antibioticus* or *Nocardia lactamdurans*. The acyltransferase gene may originate from *P. chrysogenum*, *P. nalgiovense* or *A. nidulans*.

In a preferred embodiment, a penicillin producing fungal strain is used which recombinantly expresses expandase. More preferably, a fungus of the genus *Aspergillus* or *Penicillium* is used, most preferably a strain of *Penicillium chrysogenum*. *P. chrysogenum* strain Panlabs P14-B10, DS 18541 (deposited at CBS under accession number 455.95) is an example of a suitable host for expandase expression.

The construction of recombinant expandase-expressing strains is within the knowledge of the skilled person. Examples of expression cassettes which can be used for the construction of recombinant expandase-expressing fungal strains are disclosed in EP-A-0532341, Crawford *et al.* (Biotechnol. 13 (1995), 58-62) and WO95/04148. Care should be taken to select a transformed strain which has a sufficiently high level of expandase expression. Such transformants can for instance be selected by testing their capacity to produce adipyl-7-ADCA as described by Crawford *et al.* (*supra*).

In a different embodiment, a cephalosporin-producing strain is used which recombinantly expresses acyltransferase, for instance an acyltransferase-producing *Acremonium chrysogenum* strain. An *A. chrysogenum* strain recombinantly expressing acyltransferase will thereby produce an acyl-7-ACA derivative, since such a strain natively expresses hydroxylase and acetyltransferase.

The present invention further describes a process for the recovery of an acyl-7-cephalosporin derivative from the fermentation broth of a microbial fermentation according to the invention using specific solvents, e.g. the recovery of an acyl-7-ADCA derivative, such as adipyl-, pimelyl, 2-(carboxyethylthio)acetyl-, 3-carboxymethylthio)propionyl- or trans β-hydromuconyl-7-ADCA, from the fermentation broth of an expandase-expressing *P. chrysogenum* strain.

Specifically, a 7-acylated cephalosporin derivative is recovered from the fermentation broth by extracting the broth filtrate with an organic solvent immiscible with water at a pH of lower than about 4.5 and back-extracting the same with water at a pH between 4 and 10.

The broth is filtered and an organic solvent immiscible with water is added to the filtrate. The pH is adjusted in order to extract the 7-acylated cephalosporin derivative from the aqueous layer. The pH range has to be lower than 4.5; preferably between 4 and 1, more preferably between 2 and 1. In this way, the 7-acylated cephalosporin derivative is separated from many other impurities present in the fermentation broth. Preferably a smaller volume of organic solvent is used, e.g. half the volume of solvent relative to the volume of aqueous layer, giving a concentrated solution of 7-acylated cephalosporin derivative, so achieving reduction of the volumetric flow rates. A second possibility is whole broth extraction at a pH of 4 or lower. Preferably the broth is extracted between pH 4 and 1 with an organic solvent immiscible with water.

Any solvent that does not interfere with the cephalosporin molecule can be used. Suitable solvents are, for instance, butyl acetate, ethyl acetate, methyl isobutyl ketone, alcohols like butanol etc.. Preferably 1-butanol or isobutanol are used.

Hereafter, the 7-acylated cephalosporin derivative is back extracted with water at a pH between 4 and 10, preferably between 6 and 9. Again the final volume can be reduced. The recovery can be carried out at temperatures between 0 and 50°C, and preferably at ambient temperatures.

The 7-acylated cephalosporin derivatives produced by the process of the invention are conveniently used as an intermediate for the chemical synthesis of semisynthetic cephalosporins, since the 7-aminogroup is adequately protected by presence of an appropriate acyl side chain.

Alternatively, the 7-acylated cephalosporin derivatives are deacylated in a one-step enzymatical process, using a suitable enzyme, e.g. *Pseudomonas* SE83 acylase.

Preferably, an immobilized enzyme is used, in order to be able to use the enzyme repeatedly. The methodology for the preparation of such particles and the immobilization of the enzymes have been described extensively in EP-A-0222462. The pH of the aqueous solution has a value of, for example pH 4 to pH 9, at which the degradation reaction of cephalosporin is minimized and the desired conversion with the enzyme is optimized. Thus, the enzyme is added to the aqueous cephalosporin solution while maintaining the pH at the appropriate level by, for instance, adding an inorganic base, such as a potassium hydroxide solution, or applying a cation exchange resin. When the reaction is completed the immobilized enzyme is removed by filtration. Another possibility is the application of the immobilized enzyme in a fixed or fluidized bed column, or using the enzyme in solution and removing the products by membrane filtration. Subsequently, the reaction mixture is acidified in the presence of an organic solvent immiscible with water. After adjusting the pH to about 0.1 to 1.5, the layers are separated and the pH of the aqueous layer is adjusted to 2 to 5. The crystalline N-deacylated cephalosporin is then filtered off.

The deacylation can also be carried out chemically as known in the prior art, for instance via the formation of an imino-chloride side chain, by adding phosphorus pentachloride at a temperature of lower than 10°C and subsequently isobutanol at ambient temperatures or lower.

Example 1

Fermentative production of acyl-7-ADCA

P. chrysogenum strain Panlabs P14-B10, deposited at CBS under the accession number 455.95, is used as the host strain for the expandase expression cassette constructs.

The expression cassette used containing the expandase gene under the *P. chrysogenum* IPNS gene transcriptional and translational regulation signals is described in Crawford *et al.* (*supra*). Transformation and culturing conditions are as described in Crawford *et al.* (*supra*). Transformants are purified and analyzed for expression of the expandase enzyme by testing their capacity to produce adipyl-7-ADCA as described by Crawford *et al.* (*supra*).

Acyl-7-ADCA producing transformants are inoculated at 2.106 conidia/ml into a seed medium consisting of (g/l): glucose, 30; Pharmamedia (cotton seed meal), 10; Corn Steep Solids, 20; $(NH_4)_2SO_4$, 20; $CaCO_3$, 5; KH_2PO_4, 0,5; lactose, 10; yeast extract, 10 at a pH before sterilisation of 5.6.

The seed culture (20 ml in 250 ml Erlemeyer closed with a cotton plug) is incubated at 25°C at 220 rpm. After 48 hours, 1 ml was used to inoculate 15 ml of production medium consisting of (g/l): KH_2PO_4, 0,5; K_2SO_4, 5; $(NH_4)_2SO_4$, 17,5; lactose, 140; Pharmamedia, 20; $CaCO_3$, 10; lard oil, 10 at a pH before sterilisation of 6.6.

After inoculation with the seed culture, a 20% stock solution of the precursor of choice, adjusted to pH 6.5 with KOH, is added to the fermentation to reach a final concentration of 0.5%.

The production culture is cultured at 25°C and 220 rpm for 168 hours in a 250 ml Erlemeyer flask closed with a milk filter. Evaporated water is replenished every other day.

At the end of the production fermentation, the mycelium is removed by centrifugation or filtration and acyl-7-ADCA is analyzed by HPLC.

Example 2

Analysis of acyl-7-ADCA production

Fermentation products from transformed *Penicillium* strains were analyzed by high performance liquid chromatography (HPLC). The HPLC system consisted of the following components: P1000 solvent delivery system (TSP), Autosampler model basic marathon (Spark Holland) (injection volume 3), UV150 (TSP) variable wavelength detector (set at 260 nm) and a PC1000 datasystem (TSP). The stationary phase was a YMC pack ODS AQ 150*4.6 mm column. The mobile phase consisted of 84% phosphate buffer pH 6.0, to which 0.17% tetrabutylammonium hydrogen sulfate has been added, and 16% acetonitril. The products were quantitated by comparison to a standard curve of the expected acyl-7-ADCA.

Example 3

Identity of acyl-7-ADCA products

A recombinant expandase-expressing *P. chrysogenum* strain was cultured according to Example 1 in the presence of the following precursors each: adipic acid, suberic acid, sebacic acid, pimelic acid and azelaic acid.

Analysis according to Example 2 of the fermentation products of these fermentations showed that fermentation in the presence of adipic acid, suberic acid and sebacic acid resulted in the formation of adipyl-7-ADCA, whereas pimelyl-7-ADCA was formed in case pimelic acid or azelaic acid were fed.

When high concentrations of suberic acid were used during fermentation (2.0% instead of 0.5%), a small but significant amount of suberyl-7-ADCA was detected next to adipyl-7-ADCA.

Claims

1. A process for the production of an N-deacylated cephalosporin compound comprising the steps of:

* fermenting a microbial strain capable of β-lactam production and expressing acyltransferase as well as expandase activity, and optionally acetyltransferase and/or hydroxylase activity, in the presence of a side chain precursor according to formula (1)

$$HOOC\text{-}X\text{-}(CH_2)_n\text{-}COOH \qquad (1)$$

wherein

n is an even number of at least 2, and

X is $(CH_2)_p\text{-}A\text{-}(CH_2)_q$, wherein

p and q each individually are 0, 1, 2, 3 or 4, and

A is CH=CH, C≡C, CHB, C=O, O, S, NH, the nitrogen optionally being substituted or the sulfur optionally being oxidized, and B is hydrogen, halogen, C_{1-3} alkoxy, hydroxyl, or optionally substituted methyl, with the proviso that p+q should be 2 or 3, when A is CH=CH or C≡C, or p+q should be 3 or 4, when A is CHB, C=O, O, S or NH,

or a salt, ester or amide thereof, said side chain precursor yielding a acyl-6-APA derivative, the acyl group having a structure according to formula (2)

$$HOOC\text{-}X\text{-}CO\text{-} \qquad (2)$$

wherein X is defined as above,

said acyl-6-APA derivative being *in situ* expanded to the corresponding acyl-7-ADCA derivative, and optionally further reacted to the acyl-7-ADAC or acyl-7-ACA derivative,

* recovering the acyl-7-cephalosporin derivative from the fermentation broth
* deacylating said acyl-7-cephalosporin derivative, and
* recovering the crystalline 7-cephalosporin compound.

2. The process of claim 1, wherein a side chain precursor according to formula (1) is used wherein n is an even number of at least 2, and X is $(CH_2)_p$-A-$(CH_2)_q$, wherein p is 1, q is 2 and A is CH_2.

3. The process of claim 2, wherein the side chain precursor is suberic acid or sebacic acid.

4. The process of claim 1, wherein a side chain precursor according to formula (1) is used wherein n is an even number of at least 2, and X is $(CH_2)_p$-A-$(CH_2)_q$, wherein p and q are 2 and A is CH_2.

5. The process of claim 4, wherein the side chain precursor is azelaic acid.

6. The process of any one of the claims 1 to 5, wherein the microbial strain is a penicillin-producing strain provided with an expression cassette providing for expandase expression.

7. The process of claim 6, wherein the penicillin-producing strain is *Penicillium chrysogenum*.

8. The process of claim 6 or 7, wherein the crystalline cephalosporin compound is 7-ADCA.

9. The process of any one of the claims 1 to 5, wherein the microbial strain is a cephalosporin-producing strain provided with an expression cassette providing for acyltransferase expression.

10. The process of claim 9, wherein the cephalosporin-producing strain is *Acremonium chrysogenum*.

11. The process of claim 9 or 10, wherein the crystalline cephalosporin compound is 7-ACA.

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
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According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

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Documentation searched other than minimumdocumentation to the extent that such documents are included in the fields searched

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of theinternational search	Date of mailing of the international search report
3 September 1998	10/09/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Delanghe, L

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